Pain 74 (1998) 75–81

Peripheral antinociceptive effect of an adenosine kinase inhibitor, with augmentation by an adenosine deaminase inhibitor, in the rat formalin test

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Received 13 May 1997; revised version received 2 September 1997; accepted 11 September 1997

Abstract

This study examined the ability of an adenosine kinase inhibitor (5′-amino-5′-deoxyadenosine; NH_2dAD), an adenosine deaminase inhibitor (2′-deoxycoformycin), and combinations of these agents to produce a peripheral modulation of the pain signal in the low concentration formalin model. Drugs were administered in combination with 0.5% formalin, or into the contralateral hindpaw to test for systemic effects, and episodes of flinching behaviors determined. Coadministration of NH_2dAD 0.1–100 nmol with formalin produced antinociception as revealed by an inhibition of flinching behaviors. This action was peripherally mediated as it was not seen following contralateral administration of the NH_2dAD, and was due to accumulation of adenosine and activation of cell surface adenosine receptors as it was blocked by the adenosine receptor antagonist caffeine. Antinociception was intensity-dependent, as it was not seen when higher concentrations of formalin (0.75%, 1.5%) were used. The coadministration of the selective adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine revealed the presence of an inhibitory tone of adenosine when the intrinsic antinociceptive effect of NH_2dAD was obscured by the solvent or the stimulus intensity. 2′-Deoxycoformycin 0.1–100 nmol did not produce any intrinsic effect, but 100 nmol coadministered with low concentrations of NH_2dAD, which lacked an intrinsic effect, augmented antinociception by NH_2dAD. Again, this was a peripheral rather than a systemic response. The combined action of the adenosine kinase and deaminase inhibitors was completely reversed by coadministration of caffeine. Antinociception with NH_2dAD is observed at higher concentrations of formalin in second trial experiments. This study demonstrates a peripheral antinociceptive action mediated by endogenous adenosine which accumulates following the peripheral inhibition of adenosine kinase; this action is due to activation of an adenosine A_1 receptor.

Keywords: Adenosine kinase; Adenosine deaminase; Formalin test; Antinociception; Adenosine A_1 receptor

1. Introduction

Adenosine exerts inhibitory effects on a number of aspects of the inflammatory process by actions on a number of inflammatory cell types, and has recently been proposed to be an endogenous anti-inflammatory agent (Cronstein, 1994, 1995). Peripheral concentrations of adenosine are increased by inflammation (Cronstein et al., 1995), and anti-inflammatory actions of methotrexate and sulfasalazine, which are used clinically in the treatment of rheumatoid arthritis, appear to be mediated in part by an increased release of adenosine (Cronstein et al., 1993; Cronstein, 1995). An inhibitor of adenosine kinase which promotes a peripheral accumulation of adenosine by inhibiting the intracellular phosphorylation of adenosine (Cronstein et al., 1995) also produces anti-inflammatory actions which are primarily mediated by adenosine A_2 receptor activation (Rosengren et al., 1995).

A number of chemical mediators contribute to activation of sensory neurons in generating the pain signal in inflammation (Levine and Taiwo, 1994; Dray, 1995). Adenosine can influence initiation of the pain signal by a peripheral action at the sensory nerve terminal, with the nature of the effect depending upon the adenosine receptor subtype activated. Thus, in rodents, activation of adenosine A_1 receptors produces a peripheral antinociceptive response, while the

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0304-3959/98/$19.00
PII S0304-3959(97)00153-X

activation of adenosine A_2 receptors produces a pronociceptive, pain facilitatory effect (Taiwo and Levine, 1990; Karlsten et al., 1992; Aley et al., 1995; Doak and Sawynok, 1995). Adenosine A_1 receptor mediated antinociception results from inhibition of adenylate cyclase, while pronociceptive actions resulting from adenosine A_2 receptor activation involves stimulation of adenylate cyclase within the sensory nerve terminal (Taiwo and Levine, 1991; Khasar et al., 1995). Activation of adenosine A_3 receptors also produces a pain facilitatory effect, and this is due to release of histamine and 5-hydroxytryptamine from mast cells and a subsequent action of these amines on sensory nerve terminals (Sawynok et al., 1997). Pain enhancing actions of adenosine also have been demonstrated in human studies, but curiously, adenosine A_1 receptors have been implicated in such actions (Pappagallo et al., 1993; Gaspardone et al., 1995). Whether this represents a species difference or is due to methodological issues is not clear (see Sawynok, 1997).

Endogenous adenosine levels within the cell are regulated by the action of adenosine kinase and adenosine deaminase, with adenosine kinase having a higher affinity for adenosine than adenosine deaminase in most preparations (reviewed in Meghi, 1991). Extracellular levels of adenosine in neural tissue can be augmented by inhibition of adenosine kinase and in some cases of adenosine deaminase (Sciotti and Van Wylen, 1993; Pak et al., 1994; Lloyd and Fredholm, 1995; Golembiowska et al., 1996; White, 1996). A synergistic enhancement of release is observed following coadministration of both inhibitors in some regions in neurochemical studies (Pak et al., 1994; Golembiowska et al., 1995, 1996) and can be inferred from some behavioral studies (Murray et al., 1993; Zhang et al., 1993; Poon and Sawynok, 1996).

The purpose of the present study was to determine whether the peripheral administration of 5′-amino-5′-deoxyadenosine (NH_2dAD), an inhibitor of adenosine kinase (Miller et al., 1979), and 2′-deoxycoformycin, an inhibitor of adenosine deaminase (Agarwal et al., 1977), and of combinations of these agents, can produce a peripheral modulation of the pain signal using the low concentration formalin test. This model was chosen as it reveals peripheral regulatory effects of adenosine and a number of selective adenosine agonists administered exogenously (Karlsten et al., 1992; Doak and Sawynok, 1995). Potential actions mediated by systemic absorption were evaluated by administering drugs into the contralateral hindpaw. The involvement of adenosine in effects observed was examined by determining the ability of adenosine receptor antagonists to modify the response.

2. Methods

Experiments were conducted using male Sprague–Dawley rats supplied by Charles River, Quebec, Canada. Body weights were 120–165 g with group mean values 140–150 g. Some rats were tested 1 week later in a second trial, and at this time, group mean body weight values were approximately 200 g. Rats were housed in groups of 2–4, and maintained on a 12:12 h light/dark cycle at 22 ± 1°C. Food and water were freely available. Procedures were approved by the University Committee on Laboratory Animals.

For behavioral testing, rats were placed in a 28 × 28 × 28 cm observation chamber for an initial 20 min accommodation interval to familiarize them with their surroundings. Formalin at the indicated concentrations, individual drugs and drug combinations were injected s.c. in a volume of 50 μl into the left dorsal hindpaw of the rat. All were administered as a co-injection with the formalin. When drug injections were made into the contralateral paw, the injection immediately preceded formalin injection into the other paw. Following injections, rats were returned to the observation chamber and observed in 2 min bins for a 60 min interval (phase 1, 0–12 min; phase 2, 16–60 min) for the expression of flinching behaviors. These include lifting, shaking and overt flinching that manifests as a ripple over the haunch. These behaviors are discrete and easily quantifiable, and exhibit good reproducibility between independent observers. Two rats were observed at one time, with observations taking place in alternating 2 min bins. Behavioral scores collected in any given bin were assumed to be similar to that in the adjacent bin, but no correction for this was made. Thus, cumulative values for any given interval represent about half of the real incidence of behaviors. Data is presented in 2 min bins to depict the time course, or calculated cumulatively over the specified time interval.

Formalin 0.5–5% produces a concentration-related increase in flinch behaviors in both phase 1 and phase 2; at 0.5% phase 2, but not phase 1, responses differ significantly from saline responses (Doak and Sawynok, 1997). All results in this study refer to phase 2 responses, as this component exhibits an inflammatory component (Tjølsen et al., 1992). Most rats received formalin 0.5% but some received 0.75% or 1.5% as specified.

5′-Amino-5′-deoxyadenosine (NH_2dAD), caffeine and dimethylsulfoxide (DMSO) were obtained from Sigma, St. Louis, MO, formalin (37% formaldehyde) from British Drug Houses, Toronto, Ontario, and 8-cyclopentyl-1,3-dimethylxanthine (CPT) and 3,7-dimethyl-1-propargylxanthine (DMPX) from Research Biochemicals Inc., Natick, MA. 2′-Deoxycoformycin (Pentostatin) was a gift from Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI. All drugs (except for CPT) were dissolved in saline and diluted to the stated dose with the formalin. CPT was dissolved in a final concentration of 10% DMSO. Appropriate vehicle controls (formalin/DMSO) were included in this case.

Data was analyzed using analysis of variance followed by the Student-Newman–Keuls test for multiple groups, or the Student t-test for two groups.

3. Results

3.1. Antinociception following peripheral administration of an adenosine kinase inhibitor

The peripheral coadministration of NH_2dAD with formalin 0.5% produces a dose-dependent suppression of phase 2 flinching behaviors (Fig. 1). This effect persists for the entire duration of the second phase of the response (16–60 min following injection) (Fig. 1, inset). There were no discernable effects of NH_2dAD on motor function at any of the doses used. Antinociception is due to a peripheral effect of the adenosine kinase inhibitor within the hindpaw as it is seen following coadministration with the formalin (ipsilateral administration) but not following injection into the contralateral hindpaw (Fig. 2). This action is due to a peripheral accumulation of adenosine as it is reversed by coadministration of the non-selective adenosine receptor antagonist caffeine (Fig. 3). When the more selective adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) was used, the vehicle (10% DMSO) appeared to blunt the antinociceptive response to the adenosine kinase inhibitor; nevertheless, a significant augmentation of flinching behaviors occurred with CPT in the presence but not the absence of the inhibitor (Fig. 4). Antinociception by NH_2dAD was less readily demonstrated when higher concentrations of formalin were used (0.75%, 1.5%) (Fig. 5A). Interestingly, while there was little reduction in flinching behaviors at higher intensities of stimulation, the inclusion of CPT still revealed the presence of an inhibitory tone generated by adenosine accumulation when NH_2dAD was present (Fig. 5B). No significant effect was seen in the presence of 3,7-dimethyl-1-propargyl-xanthine (DMPX) 15 nmol (data not shown). Doses of CPT and DMPX were selected on the basis of their previously demonstrated ability to block adenosine A_1 and A_2 receptors in this paradigm (Doak and Sawynok, 1995).

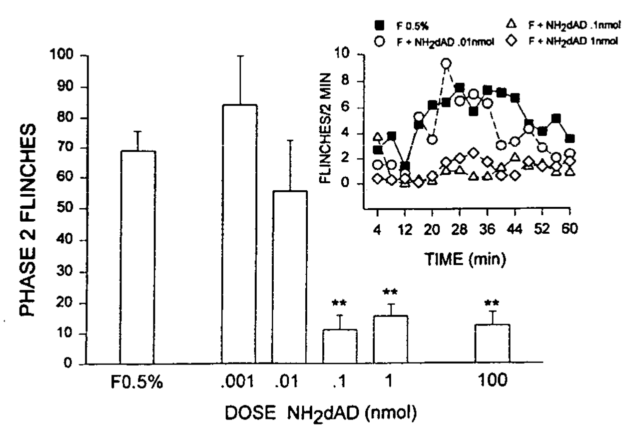

Fig. 1. Antinociceptive action of NH_2dAD following coadministration with formalin (F) 0.5% into the dorsal hindpaw of the rat. Values depict means ± SEM. SEM values not shown in the inset in the interest of clarity. n = 5–10; $^{**}P < 0.01$ compared to formalin ($F = 15.2$, $P < 0.0001$).

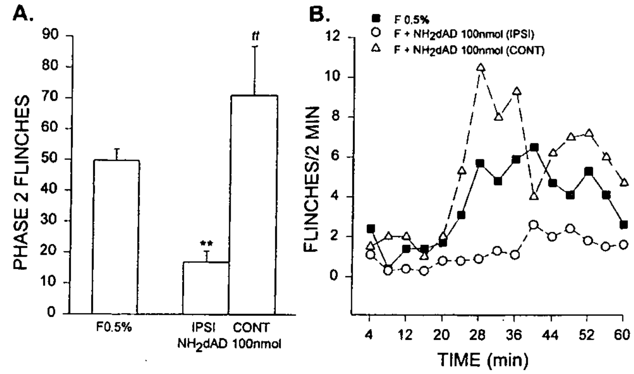

Fig. 2. Effects of ipsilateral (IPSI) and contralateral (CONT) administration of NH_2dAD with formalin (F) 0.5%. (A) Cumulative phase 2 responses, (B) time course of response. Values depict means ± SEM. n = 6–8; $^{**}P < 0.01$ compared to formalin, $^{tt}P < 0.01$ compared to IPSI ($F = 12.6$, $P < 0.0001$).

3.2. Effects of an inhibitor of adenosine deaminase

The peripheral administration of a range of doses of 2′-deoxycoformycin did not produce any significant intrinsic effect on flinching behaviors generated by formalin 0.5% (Fig. 6A). However, the coadministration of 2′-deoxycoformycin 100 nmol with low doses of NH_2dAD, which in themselves did not produce an intrinsic effect, produced a significant augmentation of antinociception by NH_2dAD (Fig. 6B). The effect of 2′-deoxycoformycin was produced peripherally, as no augmentation was seen following administration of 2′-deoxycoformycin into the contralateral hindpaw (Fig. 7). In this experiment, the enhancement of the action of NH_2dAD by 2′-deoxycoformycin was less marked because the NH_2dAD, even at these low doses, produced a significant intrinsic action. The effect of the combined action of the two agents was reversed completely by the coadministration of caffeine 500 nmol, indicating that

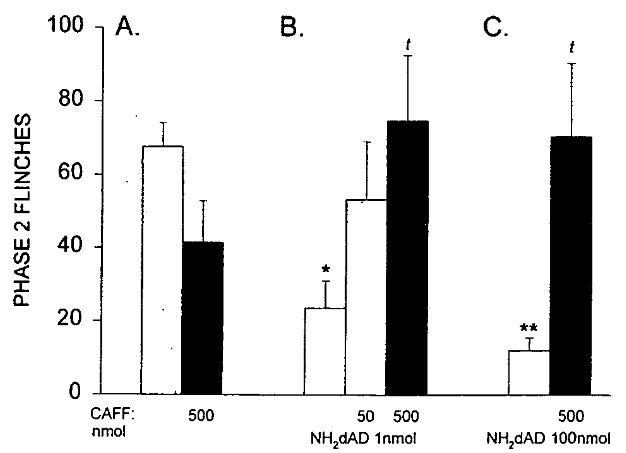

Fig. 3. Reversal of the antinociceptive action of NH_2dAD against formalin (F) 0.5% by coadministration of caffeine (CAFF). Values depict means ± SEM. n = 5–7; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to formalin, $^{t}P < 0.05$ compared to corresponding NH_2dAD group (for (B), $F = 3.99$, $P = 0.0164$; for (C), $F = 6.44$, $P = 0.0063$).

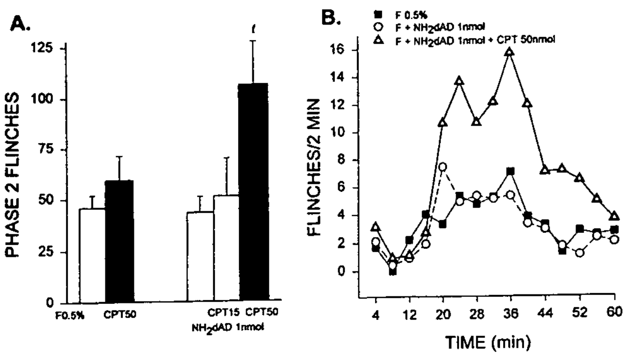

Fig. 4. Unmasking of an inhibitory tone for endogenous adenosine by CPT. (A) Cumulative responses, (B) time course of reversal. CPT was dissolved in a final concentration of 10% DMSO. Values depict means ± SEM. $n = 5$–7 per group; $'P < 0.05$ compared to NH_2dAD group ($F = 4.55$, $P = 0.0300$).

it resulted from a peripheral accumulation of adenosine (Fig. 7).

3.3. Antinociception at higher concentrations of formalin

During the course of these experiments, some rats which had received 0.5% formalin in the first trial were tested 1 week later using the opposite paw for injections. Formalin 0.5% consistently produced less effect than in the first trial, but responses to 0.75% and 1.5% were well maintained. It was noted that NH_2dAD produced antinociception with these higher formalin concentrations in the second trial. When examined directly, 100 nmol NH_2dAD produced no effect in a first trial (body weights 145 ± 2 g, $n = 12$), but did produce antinociception in the second trial (body weights 214 ± 3 g) (Fig. 8A). This effect was not restricted to this dose, as 1 and 10 nmol also were effective (Fig. 8B). Antinociception was seen also at 0.75% (Fig. 8C), and was reversed by coadministration of caffeine (Fig. 8C) or CPT (Fig. 8D).

4. Discussion

The present study demonstrates a peripheral antinociceptive action of the adenosine kinase inhibitor NH_2dAD when flinching behaviors are determined in the low concentration formalin model. The adenosine deaminase inhibitor 2′-deoxycoformycin lacks intrinsic activity, but significantly enhances the action of NH_2dAD. This profile of activity is similar to that observed following spinal administration of these agents in nociceptive tests (Keil and DeLander, 1992; Poon and Sawynok, 1995, 1996) and supraspinal administration in tests for anticonvulsant actions (Murray et al., 1993; Zhang et al., 1993). Antinociception appears due to activation of a cell surface adenosine A_1 receptor, as it is blocked by caffeine and revealed by CPT when the intrinsic effect of NH_2dAD is obscured (cf. Figs. 4 and 5). A number of previous studies using somewhat different approaches have provided evidence for a peripheral antinociceptive action due to adenosine A_1 receptor activation (Taiwo and Levine, 1990; Karlsten et al., 1992; Aley et al., 1995; Doak and Sawynok, 1995). Antinociception by NH_2dAD is observed in the second phase of the formalin test which is due to an inflammatory component and the release of a number of peripheral mediators (Tjølsen et al., 1992). Endogenous adenosine appears to be released during this phase of the formalin response as coadministration of adenosine antagonists with formalin 2.5% significantly modifies phase 2 responses (Doak and Sawynok, 1995). While little intrinsic effect with antagonists is seen at 0.5%, the presence of NH_2dAD is able to augment the amount of adenosine which accumulates under these conditions to the extent that it now produces a pharmacological response. Even under conditions when the intrinsic antinociception produced by NH_2dAD is blunted by the presence of vehicle or at higher formalin concentrations, the presence of the A_1 adenosine receptor antagonist reveals the presence of an inhibitory tone due to some adenosine accumulation.

Both adenosine kinase and adenosine deaminase are intracellular enzymes (Meghi, 1991), although there is some evidence for an extracellular adenosine deaminase (Franco et al., 1986). The adenosine which activates adenosine receptors probably originates from inhibition of adenosine kinase (and adenosine deaminase in the combination experiments) intracellularly, efflux of adenosine from the cell along a concentration gradient, and subsequent activation of adenosine A_1 receptors on the extracellular surface

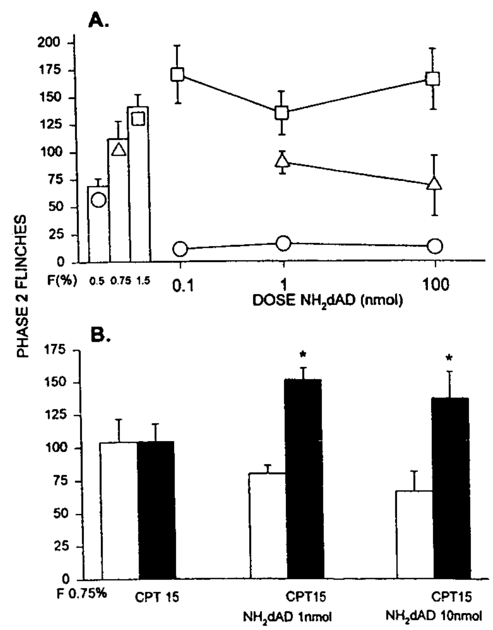

Fig. 5. Dependence of antinociceptive action of NH_2dAD on formalin concentration. (A) Lack of effect of NH_2dAD when formalin 0.75% and 1.5% are used. (B) Unmasking of inhibitory tone in the presence of NH_2dAD by CPT when formalin 0.75% is used. CPT was dissolved in a final concentration of 10% DMSO. Values depict means ± SEM. $n = 4$–7 per group; $*P < 0.05$ compared to corresponding NH_2dAD group ($F = 4.04$, $P = 0.0404$).

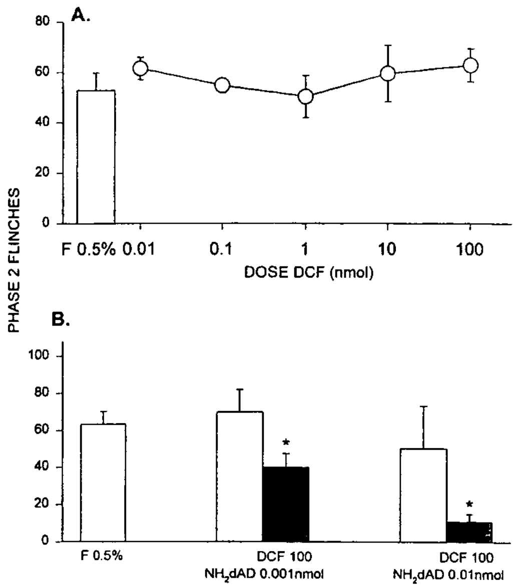

Fig. 6. (A) Lack of intrinsic effect of coadministration of 2′-deoxycoformycin (DCF) with formalin (F) 0.5%, and (B) augmentation of the action of NH$_2$dAD by coadministration of 2′-deoxycoformycin. Values depict means ± SEM. (A) n = 4–11, (B) n = 11–15; $^*P < 0.05$ compared to the corresponding NH$_2$dAD group (F = 3.13, P = 0.025).

sensory nerve terminals. The specific cellular origin of the adenosine released is not clear, but it could potentially occur from a number of different cell types including neurons, endothelial cells and neutrophils (White and Hoehn, 1991; Hourani and Hall, 1994; Cronstein, 1995). Adenosine A_1 receptors have not been directly visualized on peripheral sensory nerve endings, but adenosine exerts inhibitory actions on cell bodies of dorsal root ganglion neurons via adenosine A_1 receptors (Dolphin et al., 1986), and central terminals of primary afferent neurons appear to contain some inhibitory adenosine A_1 receptors (Santicioli et al., 1993; but see Geiger et al., 1984). It is conceivable that adenosine A_1 receptors produced by the cell body are transported both to the peripheral as well as the central aspect of the primary afferent neuron.

The present behavioral results in the periphery are in direct parallel with the spinal cord where the localized spinal administration of NH$_2$dAD, but not 2′-deoxycoformycin, produces an antinociceptive action in thermal threshold (Keil and DeLander, 1992) and in inflammatory pain tests (Poon and Sawynok, 1995, 1996). Adenosine kinase inhibitors have been shown directly to augment spinal cord extracellular levels of adenosine, an effect enhanced in the co-presence of an adenosine deaminase inhibitor (Golembiowska et al., 1995, 1996). However, the present action is a peripheral action rather than being due to an effect of adenosine within the spinal cord as it is (a) seen only following coadministration of the adenosine kinase inhibitor with the formalin, but not following administration into the contralateral hindpaw, and (b) augmented only by the local but not the contralateral administration of 2′-deoxycoformycin. This latter point was important to examine, as 2′-deoxycoformycin is a potent inhibitor of adenosine deaminase, and the 100 nmol (215 μg/kg) dose used here is sufficient to produce some degree of inhibition of central adenosine deaminase activity (Padua et al., 1990). The involvement of cell surface adenosine receptors in antinociception produced by the combination of adenosine kinase and adenosine deaminase inhibition is attested to by the complete reversal of the effect by coadministration of caffeine. The dose of caffeine used (500 nmol, 1.5 mg/kg) was without intrinsic effect on flinching behaviors; this dose is lower than doses which are active systemically in the formalin 1.5% test (Sawynok and Reid, 1996).

The peripheral antinociceptive effect produced by the adenosine kinase inhibitor exhibits a dependence on stimulus intensity as it is seen primarily at a low but not at higher concentrations of formalin when examined in naive animals. However, an effect is seen at higher formalin concentrations when examined in a second trial when rats are somewhat larger, when the concentration-intensity relationship may differ somewhat. The intensity-dependence in the initial trial is reminiscent of the antinociception seen following the spinal administration of an adenosine kinase inhibitor (Poon and Sawynok, 1995) and of caffeine, whose action also depends on an endogenous tone provided by adenosine (Sawynok and Reid, 1996). It may be that at higher concentrations of formalin, higher peripheral levels of adenosine are produced; these then activate additional peripheral receptors such as adenosine A_2 and A_3 receptors. Both of these receptors produce pronociceptive actions at peripheral sites, although their actions may not necessarily be direct ones on the nerve terminal itself (Taiwo and Levine, 1990; Karlsten et al., 1992; Doak and Sawynok, 1995; Sawynok et al., 1997). Activation of these additional receptor popula-

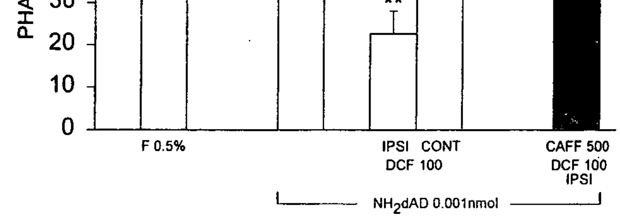

Fig. 7. Lack of augmentation of the effect of NH$_2$dAD by the contralateral administration of 2′-deoxycoformycin (DCF), and caffeine (CAFF) reversal of the combination effect of an adenosine kinase and an ipsilateral injection of an adenosine deaminase inhibitor. Values depict means ± SEM. CONT, contralateral; IPSI, ipsilateral. $^*P < 0.05$, $^{**}P < 0.01$ compared to formalin (F), $^{t}P < 0.05$, $^{tt}P < 0.01$ compared to NH$_2$dAD administered ipsilaterally with the formalin (F = 6.38, P = 0.0012).

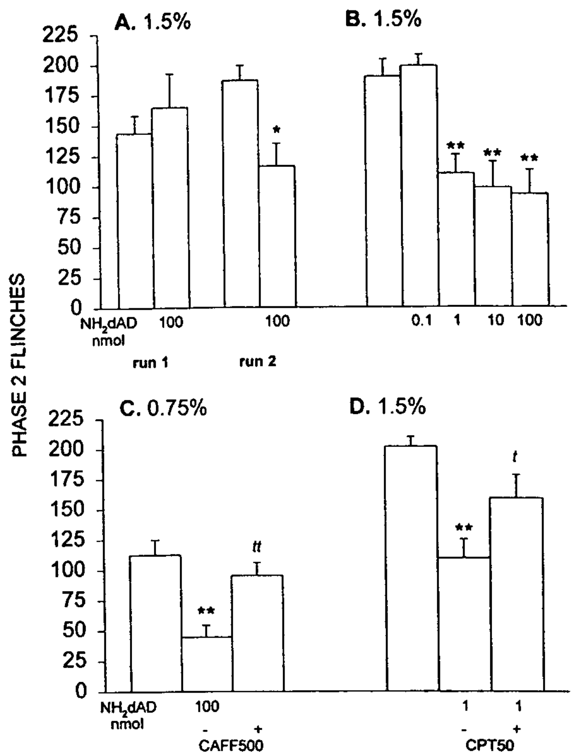

Fig. 8. Antinociceptive action of NH_2dAD with higher concentrations of formalin (1.5% and 0.75%) in a second trial. (A) Repeated testing of a single group. (B) Dose-response relationship for NH_2dAD ($F = 9.63$, $P < 0.0001$). Reversal by coadministration of (C) caffeine (CAFF) ($F = 9.75$, $P = 0.0026$) and (D) CPT (in 10% DMSO) ($F = 8.37$, $P = 0.0041$). Values depict means ± SEM. $n = 5$–7 per group; $^*P < 0.05$, $^{**}P < 0.01$ compared to formalin group, $^{t}P < 0.05$, $^{tt}P < 0.01$ compared to corresponding NH_2dAD group.

tions could counter the inhibitory action of the adenosine A_1 receptor. Alternatively, adenosine A_1 receptor activation may only be able to exert modulatory actions at mild intensities of sensory nerve stimulation with corresponding patterns of primary afferent discharge. The actions of adenosine may well occur in concert with other peripheral inflammatory mediators, e.g., substance P (Gaspardone et al., 1994) and 5-hydroxytryptamine (Doak and Sawynok, manuscript in preparation), and the intensity of stimulation would likely affect the amount and perhaps pattern of corresponding mediators available to regulate the response. The effect of adenosine on the pain signal at its point of initiation in inflammatory conditions has the potential to be complex, as it depends on the nature of the receptor activated, as well as on the degree of inflammation and consequent involvement of other mediators.

Adenosine based pharmaceuticals have therapeutic potential in a number of areas (reviewed in Williams, 1993). There has been considerable interest in the potential for adenosine agents to represent a novel class of analgesic agents, as adenosine analogs are effective in a range of nociceptive, inflammatory and neuropathic pain tests in animal models, and adenosine (i.v. infusions) and a stable analog (given spinally) have been shown to produce pain relieving properties in humans (reviewed in Sawynok, 1997). The therapeutic potential of adenosine analogs may be limited by side-effects such as hypotension, sedation or motor effects. Adenosine kinase inhibitors as a class of agents exert pain inhibiting actions both at the peripheral nerve terminal (this study) and at the level of the spinal cord (Keil and DeLander, 1992; Poon and Sawynok, 1995, 1996), and both actions likely contribute to their efficacy following systemic administration (Poon et al., 1997). While antinociception following spinal administration clearly has been observed in the absence of motor effects, it should be noted that motor effects do occur at higher doses of adenosine kinase inhibitors (Keil and DeLander, 1992; Poon and Sawynok, 1996). Given that anti-inflammatory effects of adenosine are expressed at peripheral sites in the absence of hemodynamic effects (Cronstein et al., 1995), and peripheral antinociceptive properties occur without motor changes, adenosine kinase inhibitors (perhaps peripherally acting) have the potential to represent a useful class of agents for therapeutic development. It should be noted that anti-inflammatory effects are due primarily to adenosine A_2 receptor activation (reviewed in Cronstein, 1995), while antinociception (mediated both peripherally and spinally) is due to adenosine A_1 receptor activation (reviewed in Sawynok, 1997), so manipulation of endogenous levels of adenosine that have the potential to simultaneously activate both receptor populations would be of particular benefit.

Acknowledgements

This work was supported by the Medical Research Council of Canada and a contribution from Abbott Laboratories. We thank Parke-Davis for their generous supply of 2′-deoxycoformycin (Pentostatin).

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